Hypertonic Saline Resuscitation Restores Inflammatory Cytokine Balance in Post-Traumatic Hemorrhagic Shock Patients

Shawn G Rhind[1], Sandro B Rizoli[2], Pang N. Shek[1], Kenji Inaba[2], Dennis Filips[2], Homer Tien[2], Fred Brenneman[2] & Ori D Rotstein[3]

[1]Defence Research & Development Canada (DRDC) – Toronto, Toronto, Ontario, M3M 3B9
[2]Sunnybrook & Women's College Health Sciences Centre, Dept of Surgery & Critical Care Medicine,
[3]University Health Network, Toronto General Hospital, Dept of Surgery, University of Toronto, Canada

E-mail: shawn.rhind@drdc-rddc.gc.ca

SUMMARY

Fluid resuscitation can often exacerbate injury sustained during hemorrhagic shock and is associated with altered immuno-inflammatory events. Early monocyte dysregulation and excessive proinflammatory cytokine production are thought to play a key role in the development of post-traumatic multiorgan dysfunction in resuscitated trauma patients. Compared with standard isotonic crystalloid resuscitation using 0.9% normal saline (NS), 7.5% hypertonic saline with 6% dextran-70 (HSD) has been shown in experimental studies to reduce shock/resuscitation-induced inflammatory reactions and lessen organ dysfunction. However, the immunomodulatory capacity of HSD, has not been evaluated in clinical human trials. In this prospective, randomized controlled trial we show that a single (250 mL) bolus infusion of HSD in hemorrhagic trauma patients restores the balance between pro and antiinflammatory mediators in the early post-resuscitative period. Flow cytometric single-cell analyses revealed that, compared to standard resuscitation with NS, which selectively expands the proinflammatory CD14+CD16+ monocyte phenotype, initial treatment with HSD elicits selective depletion of CD14+CD16+ cells and down-regulates monocytic adhesion molecule expression. Moreover, HSD significantly inhibits intracellular TNF- production by CD14+CD16+ monocytes, while upregulating both IL-10 and IL-1ra by CD14++CD16– monocytes. This differential profile of monocytic cytokine expression in response to HSD appears to be mediated, at least partly, by attenuation of post-resuscitation noradrenergic-stimulated signaling pathways. These findings demonstrate that HSD promotes a more balanced early inflammatory response in resuscitated hemorrhagic shock patients.

1.0 INTRODUCTION

Hemorrhagic shock is the major cause of death on the battlefield [1]. Of those soldiers wounded in combat who die of wounds, it is estimated that 20% could be salvaged before exsanguination if provided with appropriate medical care [2]. Along with urgent control of bleeding, intravenous fluid replacement therapy for intravascular volume restoration and organ perfusion is routinely required to treat combat casualties [3]. Similarly, despite advances in modern surgical care, major trauma with associated hemorrhagic shock remains a leading cause of civilian mortality in young adults [4,5]. Those patients who survive the initial tissue injury and circulatory shock are at high risk for development of the systemic inflammatory response and delayed multiorgan dysfunction syndromes [6]. Although research findings document a link between post-traumatic complications and immune dysregulation [7], the underlying cellular and molecular defects associated with the pathophysiology of resuscitated hemorrhagic shock are not fully elucidated [8].

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Adequate intravenous fluid administration for restoration of intravascular volume and maintenance of tissue perfusion is essential for successful management of hemorrhagic shock [9]. Yet, considerable controversy persists over the ideal fluid formulation [10]. The concept that early, aggressive high-volume resuscitation is critical to the optimal treatment of hemorrhagic shock has been widely accepted and practiced since the Vietnam War. Subsequently, the practice of large volume isotonic crystalloid resuscitation became the standard-of-care for civilian trauma. However, the impact of different fluid replacement strategies on shock/resuscitation-induced immunological perturbations has not been prospectively studied in trauma patients [11]. In particular, the early post-resuscitation dysfunctional period represents a critical time point that remains under-investigated, but amenable to appropriate therapeutic interventions aimed at restoring physiologic hemodynamics and reestablishing inflammatory equilibrium [12].

Various immunoinflammatory alterations have been described in clinical and experimental investigations of post-traumatic hemorrhagic shock [13]. The initial immunological response to trauma/hemorrhage is characterized by excessive innate immune stimulation, with intense monocyte/macrophage activation [7] and production of numerous inflammatory mediators [14]. The proinflammatory cytokines TNF-α and IL-1β are the primary endogenous mediators of acute inflammation, eliciting endothelial activation, capillary leak and circulatory collapse [15]. While localized inflammatory cytokine release is considered a protective host response to injury or infection [16], overproduction and loss of compartmentalization leads to unabated systemic inflammation and subsequent mulitorgan dysfunction [7]. Consequently, the proinflammatory response is tightly regulated by an intricate network of endogenous counter-inflammatory molecules [16]. Chief among these are the antiinflammatory cytokines and specific receptor antagonists, including, IL-10 and IL-1(ra) receptor antagonist [17].

In addition to direct feedback loops within the immune system, a central role of the neuroendocrine system in regulating the magnitude and duration of the post-traumatic inflammatory cascade is strongly indicated [18]. The sympathoadrenal system is rapidly activated in response to severe trauma/hemorrhage, resulting in greatly augmented (50- to 100-fold) concentrations of circulating catecholamines [19], which are themselves potent regulators of inflammatory cytokine production [18]. Monocyte/macrophages are a main target of both epinephrine (Epi) and norepinephrine (NE) induced sympathetic effects, and differentially modulate cellular activities via alterations in α and β-adrenoreceptor-stimulated intracellular signaling pathways [20].

Standard-of-care resuscitation of hemorrhagic trauma patients calls for prompt replacement of intravascular fluid using large volumes (2–3 times blood loss) of isotonic crystalloids, such as normal saline (0.9% NaCl) [21]. While aggressive isotonic fluid administration adequately restores systemic blood pressure and is lifesaving in many patients, it often leads to volume overload and post-resuscitative complications [22]. These include increased blood loss and pulmonary edema, ultimately causing greater morbidity and mortality [4]. Moreover, convincing experimental evidence indicates that conventional large-volume fluid resuscitation can exacerbate shock-induced microcirculatory dysfunction and inflammatory tissue injury [3,12]. Thus, the search continues for fluid replacement regimens, which avoid intravascular fluid overload and help prevent pathophysiologic alterations induced by shock/resuscitation [9].

Small-volume hypertonic/hyperoncotic solutions appear promising as initial fluid replacements [5,23]. One of the most common formulations used for resuscitation of hemorrhagic shock is 7.5% hypertonic saline, in combination with the hyperoncotic colloid, 6% dextran-70 (*HSD*). Although hypertonic saline has been investigated for many years [24], contemporary interest arose in 1980 when Velasco *et al.* [25] demonstrated that a bolus injection of 7.5% hypertonic saline (4 mL/kg) rapidly restored intravascular volume and central hemodynamics in severely hemorrhaged dogs. Since then, the physiological responses to hypertonic resuscitation have been extensively studied in experimental animals and humans [26,27]. The immediate

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circulatory effects of hypertonic saline derive from rapid osmotic mobilization of endogenous fluids from the extravascular to the intravascular compartment; the resultant 3–4-fold expansion of plasma volume improves mean arterial pressure, cardiac output and peripheral tissue perfusion [23,27]. Overall hypertonic resuscitation results in lower fluid requirements and normalization of physiological parameters.

Since the original human study by De Filippe *et al.* [28] revealed the substantial hemodynamic benefits of hypertonic saline infusion in refractory hypovolemic trauma patients, numerous clinical reports have confirmed the efficacy and safety of hypertonic solutions for primary resuscitation of hemorrhagic shock [26]. Although limited by small sample size, the majority of prospective randomized controlled trials where patients received initial treatment with *HSD* have shown a tendency for improved early (24 h) and long-term survival to discharge, as compared to standard-of-care [5,23,29]. Similarly, a meta-analysis [30] and cohort analysis [31] of individual patient data performed by Wade *et al.*, demonstrated enhanced patient survival using *HSD* for post-traumatic resuscitation. Moreover, other studies have shown that *HSD* reduces both fluid requirements [32] and incidence of post-resuscitation complications, such as renal failure, coagulopathies, and acute respiratory distress syndrome (ARDS) [33].

More recently, the focus of shock/resuscitation research has shifted from hemodynamic restoration to the potential role of hypertonic solutions as immunomodulatory agents [5,34]. Experimental evidence from in vitro studies and animal models of hemorrhagic shock suggest that physiological increases of plasma tonicity can attenuate inflammatory reactions and improve post-resuscitative outcome [12,34,35]. For example, hypertonic saline suppresses multiple leukocyte functions, including, neutrophil-endothelial cell adhesion, cellular rolling/transmigration, and oxidative burst [36]. Consequently, hypertonic saline is more effective than isotonic fluids in protecting against neutrophil-mediated pulmonary and hepatic injury in animals after resuscitation [37,38]. Less is known concerning the modulation of monocyte/macrophage function by hypertonicity, but recent studies indicate that hypertonic resuscitation can dampen unrestrained proinflammatory cytokine cascades, while augmenting counter-inflammatory reactions [37,39].

Much of the past research on hypertonic saline has been conducted on animals or in isolated human blood cultures. Unfortunately, extrapolation from animal studies and laboratory experiments to the clinical situation is difficult as animal responses do not wholly parallel those of humans, and since *in vivo* immunocompetant cells experience bidirectional communication with hormones and cytokines [35,40]. Thus, despite compelling experimental findings, *HSD* has not yet received widespread clinical acceptance, and its potential immunomodulatory actions remain untested in trauma patients. The present study was designed to prospectively compare the immunoinflammatory effects of initial resuscitation with a single 250-mL infusion of *HSD* followed by isotonic crystalloids versus standard-of-care only. Specifically we evaluated serial changes in: (1) intracellular expression of pro (IL-1β, TNF-α) and antiinflammatory cytokines (IL-1ra and IL-10) by blood monocytes; (2) surface expression of monocytic adhesion molecules (CD11b and CD62L); and (3) circulating catecholamine concentrations (Epi and NE) during the early resuscitative course. We hypothesized that primary fluid replacement with *HSD* would promote a more balanced early inflammatory response in resuscitated trauma patients.

2.0 METHODS

2.1 Patient Selection and Study Design

This single-centre, prospective, randomized controlled trial enrolled 20 severely injured trauma patients, admitted to the surgical emergency department of Sunnybrook and Women's College Health Sciences Centre

over a 14-month period, under a policy of 'delayed informed consent' with the approval of the Institutional Review Board. Eligible patients were entered into the study at the time of presentation to the trauma centre from the scene of injury. All patients or their next of kin were then informed of their participation as soon as possible after study enrollment and permission were obtained for continued data collection and inclusion of these data into the study database.

Patients were eligible for inclusion in the study if they sustained severe trauma; had at least one recorded episode of hypotension (systolic blood pressure ≤90 mm Hg), were 16 years of age or older, had evidence of blood loss (external, thorax, abdomen, retroperitoneum), had an injury severity score (ISS) ≥15, and as per the investigator's judgment, were expected to survive for at least 24 h. Patients were excluded if they refused to participate, were admitted ≥6 h after trauma, vital signs were absent, were pregnant, or had stigmata of chronic disease. The patient characteristics at the time of admission are summarized in Table 1.

Table 1: Demographic characteristics and clinical outcomes of the study patients

		0.9% *NS*	7.5% *HSD*	*P* value
n		10	10	
Age, years		47.5 (15.9)	49.3 (16.7)	.75
Gender, male, no. (%)		9 (90%)	7 (70%)	.76
ISS		25.9 (10.3)	26.3 (11.4)	.83
Crystalloid	- pre-hospital, mL	835 (855)	2144 (1343)	.048*
	ER, mL	4542 (2758)	3689 (1865)	.28
	total first 24h, mL	8080 (2736)	7796 (3189)	.75
Blood	- pre-hospital, units	0.5 (1.16)	1.22 (1.7)	.27
	ER, units	1.56	1.5	.62
	total first 24h, units	4.36 (6.77)	2.2 (2.9)	.38
Colloids	- total first 24h, mL	696 (773)	361 (377)	.02*
Death		2 (14.3)	0	.21

Values are mean ± SD.

Upon hospital admission, patients randomly received a either single dose of 250-mL of 7.5% hypertonic saline with 6% dextran-70 (*HSD*) or the same volume of standard 0.9% isotonic crystalloid (normal saline, *NS*), administered intravenously as a bolus infusion from identical unidentified bags. Initial resuscitation was followed by further administration of isotonic fluids dosed according to individual patient needs, as recommended by Advanced Trauma Life Support® (ATLS) guidelines [21]. No patients received vasopressors or ionotropes throughout the study period. Patients were followed until hospital discharge or death.

2.2 Antibodies and Reagents

Fluorescent mouse anti-human monoclonal antibodies (mAbs) against the cell-surface epitopes CD16–FITC, CD45–PerCP and CD14–APC, cellular adhesion molecules CD11b (Mac-1 β_2 subunit)–FITC and CD62L (L-selectin)– PE, and intracellular Fast Immune™ anti-Human-cytokine mAbs, specific for IL-1-PE, IL-1ra-PE, IL-10-PE and TNF-α-PE, along with their respective isotype-matched (IgG_1 and IgG_2a) control antibodies,

were all obtained from Becton Dickinson Biosciences (BD Biosciences, San José, CA). FACS® brand Erythrocyte Lysing Solution, Permeabilizing Solution™, and CellWASH™ (optimized PBS containing 0.1% sodium azide) were also obtained from BD Biosciences. LPS (*Escherichia coli* 055:B5), paraformaldehyde and brefeldin A (BFA) were purchased from Sigma Chemical Company (St. Louis, MO). Tissue culture reagents, including complete RPMI 1640 medium and Dulbecco's PBS (pH 7.4), were purchased from GibcoBRL/Life Technologies (Grand Island, NY). All culture flasks and 12 x 75-mm polystyrene and polypropylene tubes were obtained from Falcon (BD Biosciences). Standardized preparations of *HSD* and *NS* solutions were purchased from the hospital pharmacy.

2.3 Blood Collection, Cell Culture and Stimulation Conditions

Venous blood samples (totalling 15 mL) were withdrawn serially from each patient using existing intravenous lines over five time-points: at baseline (hospital admission), and again at 1, 3, 6, and 24-h post-resuscitation. Samples for hematological and flow cytometric analyses were collected into 3-mL sterile glass Vacutainers™ (BD, Franklin Lakes, NJ), containing EDTA and sodium heparin, respectively. After sampling, specimens were kept at room temperature and transported to the lab for culture within 3 h. All blood work was performed in a laminar flow hood using sterile technique. A 2-mL aliquot of freshly sampled sodium heparin anticoagulated whole blood was treated with the Golgi transport inhibitor BFA at a final concentration of 10 μg/mL to enable intracellular cytokine accumulation. For determination of *ex vivo, de novo* cytokine producing activity, BFA-treated heparinized whole blood was further subdivided into two 1-mL aliquots, which were incubated in the presence of vehicle (RPMI) for unstimulated cytokine expression, or with an optimal concentration (1 μg/mL) of LPS for stimulated cytokine expression. Non-BFA-treated heparinized whole blood was processed in parallel for determination of unstimulated and stimulated cellular adhesion molecule expression. All samples were then cultured for 22 h at 37°C in a 5%-CO_2 humidified atmosphere.

2.4 Cell Surface and Intracellular Immunofluorescence Staining

Due to limited blood available from trauma patients and because cellular isolation techniques can artifactually activate leukocytes resulting in changes in antigen expression, a whole-blood method was selected for measuring cell-surface and intracellular antigen expression. For phenotypic characterization of monocyte subsets, 100-μL aliquots of unstimulated and LPS-stimulated blood were incubated for 15 min at room temp in the dark with saturating dilutions of fluorochrome-conjugated anti-CD45, anti-CD14 and anti-CD16 mAbs in 12 x 75-mm polystyrene tubes. Monocyte cellular adhesion molecule expression was identified by a direct immunofluorescence technique, using anti-CD11b and anti-CD62L mAbs in conjunction with anti-CD45/CD14. Appropriate class-matched isotype immunoglobulin negative control mAbs were added simultaneously to separate tubes, at identical concentrations for each assay, to detect non-specific binding. Immediately following surface staining, cells were treated for 10 min with 2 mL of 1x FACS™ Lysing Solution, resulting in simultaneous erythrocyte lysis and partial fixation of leukocytes. After centrifugation (500 x g, 5 min) and aspiration of the supernatant, specimens for surface staining were washed with 2 mL of PBS CellWASH™ (containing 1% sodium azide and 1% bovine serum albumin). Stained cells were then fixed with 300 μL of 1% paraformaldehyde and stored at 4°C until data acquisition by flow cytometer. Thereafter, specimens for intracellular cytokine detection were further treated with 500 μL of 1x FACS Permeabilizing Solution™ and incubated for 30 min at room temp with 20 μL of the corresponding fluorescent-labeled anti-Hu-cytokine mAbs [41]. After incubation, cells were washed, aspirated and resuspended in 2% paraformaldehyde. Samples were kept at 4°C in the dark until flow cytometric aquisition.

2.5 Flow Cytometric Data Acquisition and Analysis

Stained cell suspensions were acquired on a dual-laser FACSCalibur flow cytometer (BD Biosciences) calibrated for four-colour analysis. An electronic acquisition gate was set to include all monocytes according to regionalization on the basis of anti-CD45/CD14 fluorescence emission characteristics using bivariate dotplots in CellQuest™ Pro software (BD) (**Figure 1a**). Further sequential gating was performed to identify two major subpopulations of monocytes on the basis of their coexpression of the CD16 antigen (FcRγIII) as shown in **Figure 1b**. The phenotypic frequencies of $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocyte subsets were expressed as percentages of total $CD14^{+}$ monocytes. Typically, ≥5,000 $CD14^{+}$ monocyte-gated events were acquired for assessment of the frequency of cell-associated cytokines and cellular adhesion molecule expression. Relative mean fluorescence intensities [i.e., MFI of sample antigen minus MFI of isotype control, in arbitrary units (au) scaled form 0 to 10,000] of the selected monocyte adhesion molecules and intracellular cytokines, in unstimulated and LPS-stimulated cultures, were quantified using fluorescence histogram data (**Figure 1c**). Analysis gates and quadrant markers were set to define positive and negative populations according to the non-specific staining of isotype-matched negative controls. Instrument optical alignment and fluidics were verified for each cytometer run using CaliBRITE™ beads and day-to-day variability in instrument settings were monitored and adjusted with AutoCOMP™ software (BD). Absolute cell counts were obtained by multiplying the corresponding percentages of cells derived from FACS analysis by total leukocyte counts obtained from a hematology analyzer (Coulter Electronics, Hialeah, FL).

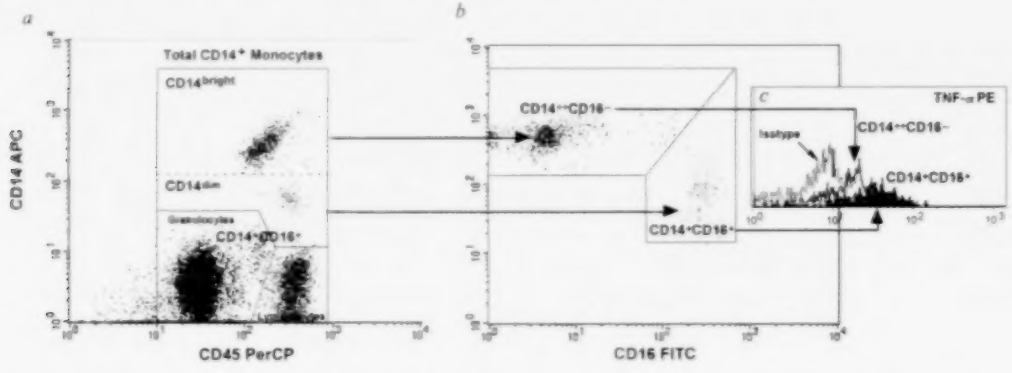

Figure 1: Representative flow cytometric immunofluorescence data: illustrating the four-colour sequential gating method used to identify whole blood monocyte subpopulations and quantify intracellular cytokine production. Leukocyte subsets (monocytes, granulocytes, lymphocytes) were distinguished according to cell-surface staining characteristics, using CD45-PerCP and CD14-APC (dotplot *a*); total $CD14^{+}$ monocytes were further classified as $CD14^{bright}$ and $CD14^{dim}$ according to their intensity of CD14 staining. Additional staining with CD16-FITC (dotplot *b*) allowed definition of $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocyte subpopulations. Panel *c* displays a corresponding single parameter fluorescence histogram (FL2) used to evaluate the %cytokine-positive and mean fluorescence intensity (MFI) of cells showing the differential pattern of TNF-α expression typical of $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocyte subsets (broken-line: isotype control antibody; solid-line: $CD14^{++}CD16^{-}$ subset; shaded histogram: $CD14^{+}CD16^{+}$ subset). The data shown are representative of a single trauma patient after LPS stimulation at 1 μg/mL for 22 h in the presence of brefeldin A (10 μg/mL).

2.6 Hormonal Analysis

Specimens for catecholamine determination were drawn into 4.5-mL vacuum tubes containing EDTA and reduced glutathione (Amersham, Arlington Heights, IL) and stored briefly on ice. Plasma was separated in a refrigerated centrifuge for 15 min (4°C; 3000 x *g*) and the supernatant frozen at –80°C until assay. Unbound catecholamine concentrations were quantitated by gas chromatography-mass spectrometry.

2.7 Statistical Analysis

Serial changes in intracellular cytokines, adhesion molecules, and hormones were evaluated by two-way ANOVA for repeated measures. Differences between resuscitation strategies were indicated by a significant treatment group x time interaction effect; the Newman-Keuls post-hoc multiple comparison test was performed to isolate specific group and time differences among treatment means using a Huynh-Feldt correction for multiple comparisons. Data within the groups were compared at each data point by a factorial ANOVA and Scheffé multiple comparison test further established detected differences. The chi-square test was used for intergroup comparisons of baseline characteristics. Unless specified otherwise, variables are expressed as means ± SE. For all comparisons, a probability of less than .05 was considered to be statistically significant.

3.0 RESULTS

3.1 HSD Prevents Expansion of the $CD14^+CD16^+$ Inflammatory Monocyte Phenotype

Peripheral blood monocytes consist of two principal subsets [42], which display distinct phenotypical and functional properties, including their cytokine production profiles [43]. We sought to determine if initial HSD resuscitation would alter the frequency of these key cellular subsets in blood samples obtained from resuscitated trauma patients. Multi-colour immunofluorescence analysis, based on the coexpression of the LPS receptor (CD14) and Fcγ receptor III (CD16), allowed the two types of monocytes to be defined: a major subset termed 'classical' monocytes, that are strongly CD14-positive but negative for CD16 ($CD14^{++}CD16^-$ monocytes) and a minor subset that are weakly CD14-positive which co-express CD16 ($CD14^+CD16^+$ monocytes). $CD14^+CD16^+$ monocytes are more mature cells that readily express proinflammatory cytokines, including TNF-α and IL-1β, but typically fail to produce significant amounts of the antiinflammatory cytokines, such as IL-10 [44]. Based on this pattern of cytokine expression, $CD14^+CD16^+$ cells have been termed 'proinflammatory' monocytes and their concentration is expanded in various pathological inflammatory states [45]. Although still within the circulation, $CD14^+CD16^+$ monocytes possess functional activities that are analogous to mature tissue macrophages [43]. A representative example of the differential monocyte subset gating procedure used for the current analysis and the staining pattern of surface expression determined from flow cytometry data is shown in **Figure 1, a–b**.

The absolute monocyte counts and relative subset distributions (as a percentage of total leukocytes) are summarized in **Table 2**. A comparison of the composition of total $CD14^+$ blood monocytes, as well as $CD14^{++}CD16^-$ and $CD14^+CD16^+$ monocyte subsets, in trauma patients resuscitated with *HSD*, demonstrated a differential pattern of monocyte subset distribution as compared to standard resuscitation with *NS*. Our results indicate that the mean (± SE) frequency (counts and proportions) of $CD14^+$ monocytes were similar at baseline and remained relatively constant throughout the experimental period in both *HSD* and *NS* resuscitated patient groups. As a percentage of total circulating monocytes, the classical $CD14^{++}CD16^-$ subset comprised the majority of circulating monocytes in both *HSD* and *NS* patient arms at baseline (77.12 ± 6.4% vs. 79.54 ± 2.24%, respectively), and the proinflammatory $CD14^+CD16^+$ subset constituted a smaller population (24.89 ±

5.29% vs. 22.32 ± 2.24%, respectively) (**Table 2**); nonetheless, the fraction of $CD14^+CD16^+$ cells was still expanded in all patients relative to reported values (~10% of monocytes) for healthy individuals [43].

	Absolute Cell Counts ($x10^9$/L) and Relative Proportions (%)							
Patient Group	Total Leukocytes	Total $CD14^+$ Monocytes		$CD14^{++}CD16^-$ Subset		$CD14^+CD16^+$ Subset		Monocyte Subset
	Counts	Counts	% Leukocytes	Counts	% Monocytes	Counts	% Monocytes	Ratio (%)[b]
Normal Saline (n =10)								
Baseline	14.75 ± 0.92	0.56 ± 0.11	3.79 ± 0.44	0.40 ± 0.10	77.12 ± 7.40	0.13 ± 0.03	24.88 ± 5.20	32.24 ± 5.22
1h	14.41 ± 1.82	0.58 ± 0.12	3.72 ± 0.43	0.41 ± 0.09	72.27 ± 1.60	0.16 ± 0.01	28.25 ± 3.26	39.10 ± 4.20
3h	12.51 ± 1.56	0.57 ± 0.09	4.86 ± 0.75	0.37 ± 0.07	67.42 ± 4.14*	0.18 ± 0.01*	31.50 ± 3.75*	48.96 ± 1.44*
6h	10.87 ± 1.07*	0.50 ± 0.08	4.87 ± 0.64	0.31 ± 0.08*	62.48 ± 5.12*	0.17 ± 0.02*	34.62 ± 4.17*	55.71 ± 6.54*
24h	11.08 ± 0.84*	0.58 ± 0.13	5.29 ± 1.13*	0.33 ± 0.10*	61.15 ± 3.35*	0.20 ± 0.01*	35.11 ± 4.69*	57.32 ± 5.71*
Hypertonic Saline (n = 10)								
Baseline	13.09 ± 0.96	0.50 ± 0.06	3.86 ± 0.57	0.39 ± 0.05	79.54 ± 2.24	0.11 ± 0.04	22.32 ± 2.24	29.23 ± 3.23
1h	12.50 ± 1.07	0.46 ± 0.05	3.00 ± 0.58	0.37 ± 0.04	84.53 ± 1.55†	0.10 ± 0.02	18.02 ± 1.63	21.33 ± 6.49
3h	12.26 ± 0.89†	0.42 ± 0.06	3.48 ± 0.56†	0.36 ± 0.09	85.22 ± 2.29*†	0.06 ± 0.02†	14.58 ± 2.29*†	17.13 ± 1.24*†
6h	10.83 ± 0.87†	0.42 ± 0.04	3.99 ± 0.46†	0.35 ± 0.03†	82.78 ± 3.10†	0.07 ± 0.01†	17.18 ± 3.09*†	20.04 ± 2.86*†
24h	9.55 ± 0.75*†	0.44 ± 0.07	4.62 ± 0.85*†	0.40 ± 0.02†	83.48 ± 7.3†	0.07 ± 0.01†	16.39 ± 5.74*†	19.52 ± 4.14*†

Table 2: Whole Blood Monocyte Counts and Subset[a] Distribution by Resuscitation Group

[a]Whole blood samples were stained simultaneously with anti-CD14 and anti-CD16 antibodies, and the percentage of $CD14^{++}CD16^-$ and $CD14^+CD16^+$ cells were determined by flow cytometric analysis within the monocyte gate. Absolute monocyte counts were obtained by automated leukocyte count, and the cell numbers of monocyte subpopulations were calculated with the percentage distribution obtained from flow cytometry. Results are expressed as mean ± SE, and statistical comparisons between treatment groups were conducted by repeated measures ANOVA.
[b]Monocyte subset ratio calculated as [(%$CD14^+CD16^+$ / %$CD14^{++}CD16^-$) x 100].
* $p < 0.05$ within trial vs. baseline; † $P < 0.05$ between trial vs. normal saline; all others, $P > 0.05$ by ANOVA.

As shown in **Table 2**, the frequency and absolute counts of circulating $CD14^+CD16^+$ proinflammatory monocytes were differentially modulated by the type of resuscitation strategy; initial resuscitation with *NS* provoked a significant ($P < .05$) expansion of the $CD14^+CD16^+$ subpopulation at 3, 6, and 24-h post-resuscitation, whereas, *HSD*-resuscitated patients exhibited a marked reduction in this subset over the same time-course. Concomitantly, the percentage and number of $CD14^{++}CD16^-$ monocytes dropped significantly ($P < .05$) in *NS* treated patients, while the proportion of these cells was modestly increased after *HSD* resuscitation. These opposing alterations in $CD14^{++}CD16^-$ and $CD14^+CD16^+$ monocyte subsets were reflected by parallel changes in the monocyte subset ratio (i.e., $CD14^+CD16^+$ / $CD14^{++}CD16^-$), which showed a progressive increase (from ~ 32 to 57) in *NS* treated patients and a decrease (from ~ 29 to 20) in *HSD* resuscitated patients (**Table 2**). These results demonstrate that *NS* resuscitation leads to a selective expansion of the proinflammatory $CD14^+CD16^+$ monocyte phenotype in the peripheral blood during the post-resuscitation period, while *HSD* elicits less overall monocyte redistribution, with a bias towards selective depletion of the $CD14^+CD16^+$ subpopulation.

3.2 HSD Resuscitation Inhibits Intracellular TNF-α Production by $CD14^+CD16^+$ Monocytes

To assess the impact of resuscitation strategy on the production of intracellular cytokines, a whole-blood multiparameter flow-cytometric assay was used to measure changes in the spontaneous and LPS-stimulated expression of pro (TNF-α, IL-1β) and antiinflammatory (IL-1ra, IL-10) cytokines in monocytes of trauma patients (**Figure 1c**). Compared to conventional cytokine assay methods (e.g., immunoassays of serum or culture supernatants), flow cytometric intracellular cytokine detection offers the unique advantages of whole blood analysis, which does not require cellular purification or isolation, and allows detection of cytokine production at the single-cell level rather than bulk systemic cytokine release [41]. For the present analysis, changes in monocytic intracellular cytokine expression were calculated as both the percentage of cytokine-positive cells (i.e., % of cells expressing cytokine of interest) and as relative MFI values (i.e., amount of cytokine produced per positive cell).

Post-traumatic shock/resuscitation-induced alterations in the spontaneous and LPS-stimulated percentage of cytokine-producing monocytes and their fluorescence intensity values are presented in **Table 3** and **Figure 2**. Upon admission, intracellular immunofluorescence analysis of unstimulated whole-blood cultures from all trauma patients measured at baseline, revealed that on average approximately 15% of $CD14^+$ blood monocytes spontaneously expressed the proinflammatory cytokines TNF-α and IL-1β with no differences between treatment arms (**Table 3**). Neither resuscitation regimen significantly affected spontaneous or stimulated expression of IL-1β, although our findings did show a trend towards higher expression of this cytokine by $CD14^+$ monocytes after *NS* resuscitation. On the other hand, spontaneous shock/resuscitation-induced expression of TNF-α by unstimulated $CD14^+$ monocytes increased significantly ($P < .05$) over time in *NS* resuscitated patients, with the % TNF-α-positive monocytes more than doubling and the MFI of positive cells increasing by almost 20% above baseline by 24-h. Importantly, *HSD* resuscitation totally reversed ($P < .05$) the spontaneous increases in TNF-α expression by unstimulated $CD14^+$ monocytes.

Table 3: Percentage (%) and mean fluorescence intensity (MFI) of cytokine-positive CD14$^+$ monocytes in unstimulated whole blood according to resuscitation treatment group

Patient Group	IL-1β		TNF-α		IL-1ra		IL-10	
	%	MFI	%	MFI	%	MFI	%	MFI
Normal Saline								
Baseline	14.8 ± 2.0	377.6 ± 20.9	14.4 ± 2.5	338.2 ± 12.1	11.9 ± 2.1	372.8 ± 17.2	10.1 ± 1.8	322.5 ± 14.1
1 h	22.1 ± 2.4	373.1 ± 18.3	17.7 ± 3.2	341.3 ±10.4	13.3 ± 3.0	379.2 ± 16.1	11.5 ± 2.9	324.4 ± 13.5
3 h	22.0 ± 2.8	382.1 ± 20.1	20.0 ± 2.8	351.8 ± 19.4*	11.6 ± 2.9	365.6 ± 15.9	11.9 ± 2.4	314.4 ± 10.5
6 h	22.9 ± 2.4	380.2 ± 27.9	21.7 ± 2.8	354.9 ± 17.6*	15.4 ± 3.3	364.2 ± 12.8	9.6 ± 2.5	312.3 ± 13.7
24 h	24.5 ± 3.2	386.3 ± 23.0	31.4 ± 3.9*	399.7 ± 19.7*	13.8 ± 3.4	358.6 ± 11.8*	14.9 ± 1.6	329.7 ± 12.3
Hypertonic Saline								
Baseline	15.20 ± 3.62	371.4 ± 11.8	13.3 ± 2.9	344.3 ± 14.1	10.2 ± 2.7	369.1 ± 20.4	9.5 ± 2.4	325.9 ± 10.1
1 h	18.51 ± 3.27	379.1 ± 8.2	16.6 ± 3.1	341.7 ± 15.6	24.2 ± 4.1*†	417.1 ± 21.9	20.1 ± 3.8*†	321.5 ± 8.5
3 h	17.24 ± 3.02	376.6 ± 11.8	17.3 ± 3.5	338.7 ± 20.4†	29.2 ± 4.8*†	415.7 ± 22.3	25.5 ± 3.0*†	368.3 ± 16.2*†
6 h	20.26 ± 5.14	375.1 ± 14.8	17.0 ± 3.6	344.2 ± 22.3†	30.7 ± 3.4*†	426.5 ± 28.7*	24.7 ± 2.0*†	357.1 ± 17.1*†
24 h	18.83 ± 2.44	375.6 ± 20.2	21.5 ± 2.8*	349.8 ± 16.7†	26.8 ± 3.3*†	447.7 ± 24.9*	21.1 ± 2.9*†	354.0 ± 25.4*†

Evaluation of monocytic cytokine production was based on the percentage and relative mean fluorescence intensity (MFI; channel ± SE) from 5,000 events within each monocyte subset. Differences in MFI for all cytokines were measured on a single parameter (FL2) histogram using a linear scale (arbitrary units scaled from 0–10,000) after subtracting MFI values from corresponding isotype-matched negative controls.
*$P < 0.05$ within trial vs. baseline; †$P < 0.05$ between trial vs. normal saline; all others, $P > 0.05$ by ANOVA

Examination of LPS-stimulated cytokine production showed a similar pattern of TNF-α expression to that observed in spontaneous cultures, with substantially augmented (>150%) production with *NS* resuscitation, as measured by changes in MFI from baseline (**Figure 2A–B**). Detailed monocyte subset analysis revealed that, although both CD14^{++}CD16$^-$ and CD14$^+$CD16$^+$ subpopulations were capable of expressing TNF-α, the expanded CD14$^+$CD16$^+$ inflammatory subset was primarily responsible for both spontaneous and LPS-induced upregulation of TNF-α production after *NS* treatment. Moreover, as shown in **Figure 2B** *HSD* resuscitation specifically inhibited TNF-α-production by CD14$^+$CD16$^+$ monocytes. These data demonstrate that *HSD* resuscitation not only reduces the mobilization of CD14$^+$CD16$^+$ monocytes to the peripheral blood of resuscitated patients, but also effectively inhibits both the spontaneous and stimulated capacity of these proinflammatory cells to produce TNF-α in response to inflammatory stimuli.

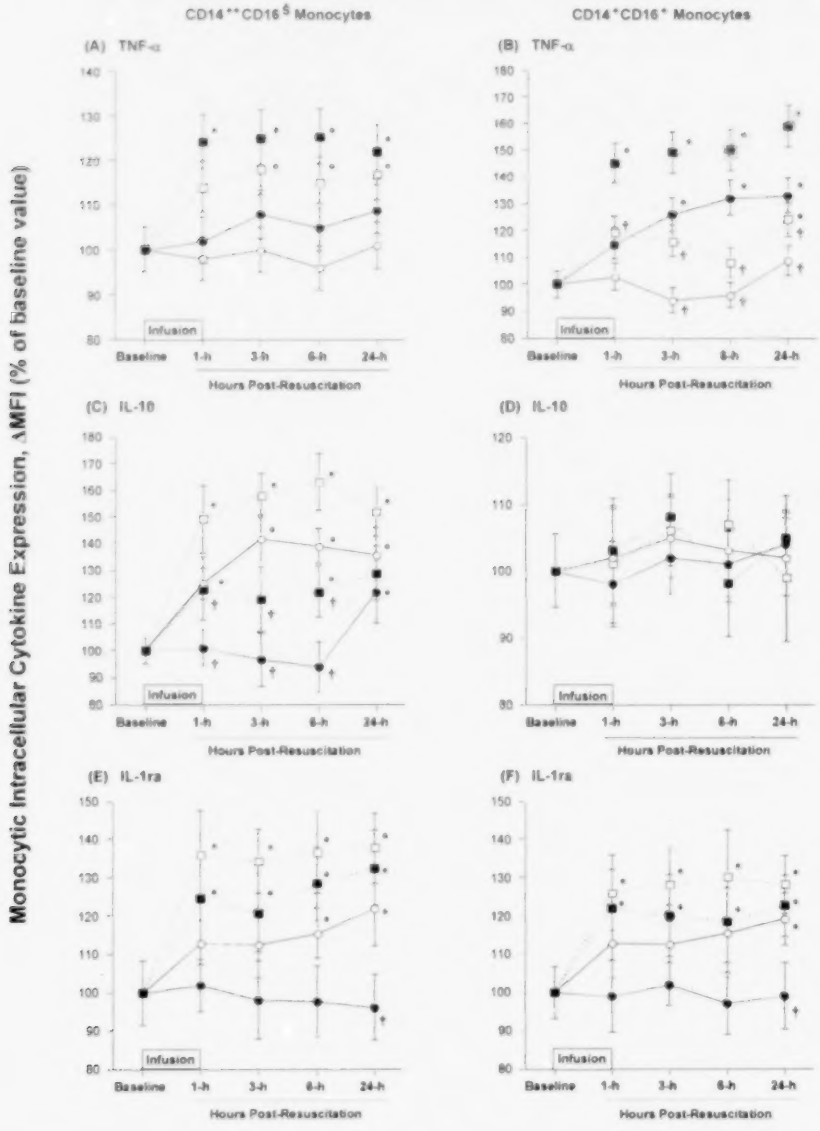

Figure 2: Changes in intracellular cytokine expression by blood monocyte subsets of resuscitated patients. Normal saline (*NS*) and hypertonic saline/dextran (*HSD*)-treated patients exhibited differential expression of tumor necrosis factor (TNF)-α (**A, B**), IL-10 (**C, D**), and IL-1ra (**E, F**) by $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ subsets, respectively. Blood was cultured without (*spontaneous*: ●, *NS*; ○, *HSD*) or with lipopolysaccharide (*LPS-stimulated*: ■, *NS*; □, *HSD*) at 1μg/mL for 20-h in the presence of brefeldin A. Intracellular cytokines were stained as described in the Methods. Data are expressed as change in mean fluorescence intensity (ΔMFI) compared to baseline (set at 100%). Significant differences: *$P < .01$, post-resuscitation values *vs.* baseline within treatment group; †$P < .01$, *HSD* vs. time-matched *NS* values.

3.3 HSD Resuscitation Enhances IL-10 and IL-1ra Production by $CD14^{++}CD16^{-}$ Monocytes

To determine the effect of hypertonicity on the ability of circulating blood monocytes to mount a counter-inflammatory response to shock/resuscitation, spontaneous and LPS-induced intracellular expression of the antiinflammatory cytokines IL-10 and IL-1ra was measured in whole blood monocyte subsets obtained from resuscitated trauma patients. As shown in **Table 3**, standard resuscitation with *NS* did not significantly influence the acute (1–6-h) spontaneous monocytic expression of either antiinflammatory cytokine, but did significantly ($P < .05$) suppress IL-1ra expression 24-h post-resuscitation. Compared to standard resuscitation, *HSD* treatment led to an early and sustained rise in spontaneous production (% and MFI) of both IL-10 and IL-1ra. In the case of IL-10, we found that the %IL-10-positive $CD14^{+}$ monocytes more than doubled by 1-h post-resuscitation and remained significantly ($P < .05$) above baseline levels after 24-h; this response was closely matched by a corresponding increase in IL-10 MFI values (**Table 3**). Importantly, monocyte subset analysis traced the *HSD*-induced increase in spontaneous IL-10 expression exclusively to augmented production by the $CD14^{++}CD16^{-}$ subset; this response was further potentiated after LPS-stimulation (**Figure 2C**). These results demonstrate the inability of $CD14^{+}CD16^{+}$ monocytes to produce IL-10 (**Figure 2D**) and are in concurrence with previously reported findings indicating that these cells fail to express significant amounts of IL-10 [44].

Correspondingly, *HSD* resuscitation also amplified IL-1ra expression in LPS-stimulated monocytes; however, in contrast to IL-10 production, both $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ subsets contributed to the overall increase in IL-1ra production (**Figure 2E-F**). Together, these observations are consistent with recent experimental animal studies [37,46] demonstrating that *HSD* can profoundly augment early post-traumatic compensatory antiinflammatory cytokine production, and that this may promote a more balanced inflammatory response, by favorably shifting the equilibrium of pro vs. antiinflammatory cytokines.

3.4 HSD resuscitation downregulates monocytic cellular adhesion molecule expression

Leukocyte recruitment and adhesion to the vascular endothelium is a pivotal event in acute inflammation and tissue damage [47]. This process requires the sequential interaction of cell-surface L-selectin (CD62L) and β_2 integrins (CD11b), with their complementary endothelial ligands. Monocyte/macrophages constitutively express low levels of these molecules, which mediate adherence (rolling and firm attachment) to activated or damaged endothelium and subsequent migration into adjacent inflamed tissues. Inappropriate upregulation of these molecules after shock resuscitation can lead to microvascular injury and the development of post-traumatic organ dysfunction [48]. In the present investigation, between 98% and 100% of all monocyte populations expressed the selected adhesion molecules at baseline, and that this proportion was not significantly altered over the sampling period by either resuscitation strategy (data not shown). Changes in monocyte surface density (expressed as % change in MFI over baseline) of CD11b and CD62L by LPS-stimulated and unstimulated blood monocytes was, however, significantly affected by the experimental treatment. On average, LPS-stimulation augmented monocytic CD11b expression by 14%. Notably, LPS-induced up-regulation of CD11b expression was significantly inhibited ($P < .05$) by treatment with *HSD* between 3 and 24-h post-resuscitation, as compared to *NS* (**Figure 3A**). Despite an apparent early rise in unstimulated CD11b expression by monocytes after *NS* resuscitation, differences were not statistically significant over time or between treatment arms.

By comparison, unstimulated CD62L expression began to rise 1-h after standard *NS* resuscitation, reaching statistical significance by 24-h ($p < .01$) (**Figure 3B**). Unstimulated CD62L levels remained unchanged following *HSD* resuscitation; however, as with CD11b, the expression of CD62L was significantly inhibited by *HSD* at 3-h, 6-h and 24-h compared with *NS*. LPS-stimulation elicited an average of 25% loss of CD62L surface expression as compared to unstimulated levels. However, no significant inter-trial treatment effects

were observed for LPS-stimulated CD62L expression over the sampling period. In sum, these findings demonstrate that both stimulated and unstimulated monocytic adhesion molecule expression is down-regulated in response to *HSD* as compared to *NS*. This suggests that *HSD* resuscitation elicits less overall monocyte activation and that at the microvascular level, *HSD* treatment may have the capacity to modulate monocyte trafficking, possibly limiting leukocyte-endothelial interactions in acute inflammatory reactions.

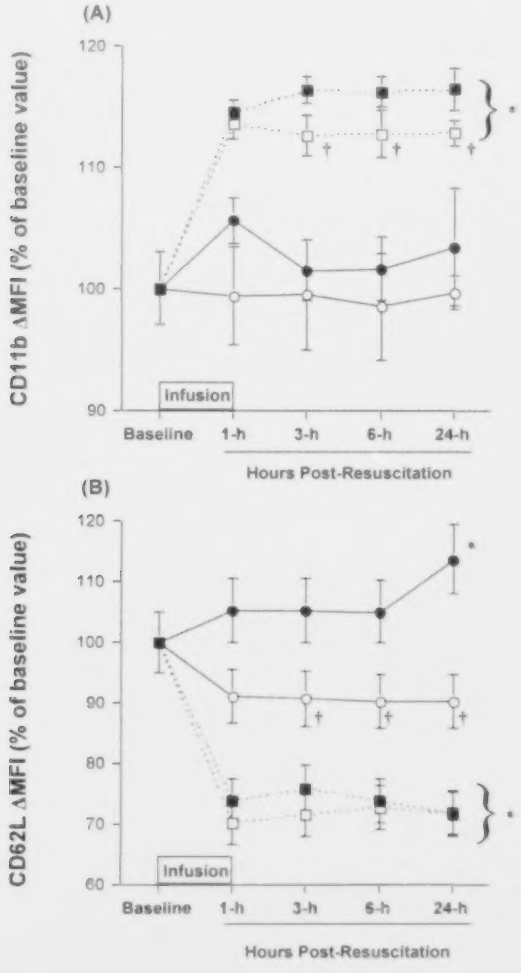

Figure 3: Changes in cellular adhesion molecule expression by blood monocytes of resuscitated trauma patients. Normal saline (*NS*) and hypertonic saline-dextran (*HSD*)-treated patients showed differential expression of CD11b (*A*) and CD62L (*B*) by unstimulated (●, *NS*; ○, *HSD*) and lipopolysaccharide-stimulated (■, *NS*; □, *HSD*) blood monocytes. Data are expressed as percentage change in mean fluorescence intensity (ΔMFI) compared to baseline conditions (set at 100%). Significant differences: $*P < .05$, post-resuscitation vs. baseline values within a treatment group; $\dagger P < 0.05$, *HSD* vs. time-matched *NS* control values.

3.5 Circulating NE Secretion is Inhibited by HSD Resuscitation

Changes in circulating concentrations of NE and Epi are shown in **Figure 4A-B,** respectively. On admission, pooled mean baseline concentrations of NE and Epi (907.9 ± 115.5 and 1207.3 ± 13.9, respectively) were substantially elevated in hemorrhagic shock patients as compared to expected basal values (reference ranges, 100–400 and 60–90 pg/mL, respectively) reported for healthy adults. Compared to baseline values, resuscitation with *NS* quickly augmented circulating NE levels up to 60% ($P < .05$) between 1 and 6-h; remarkably, *HSD* resuscitation completely abrogated ($p < .05$) the post-resuscitative elevation of NE. In contrast, Epi levels did not differ between treatment arms, but fell significantly over the post-resuscitation period in both patient groups.

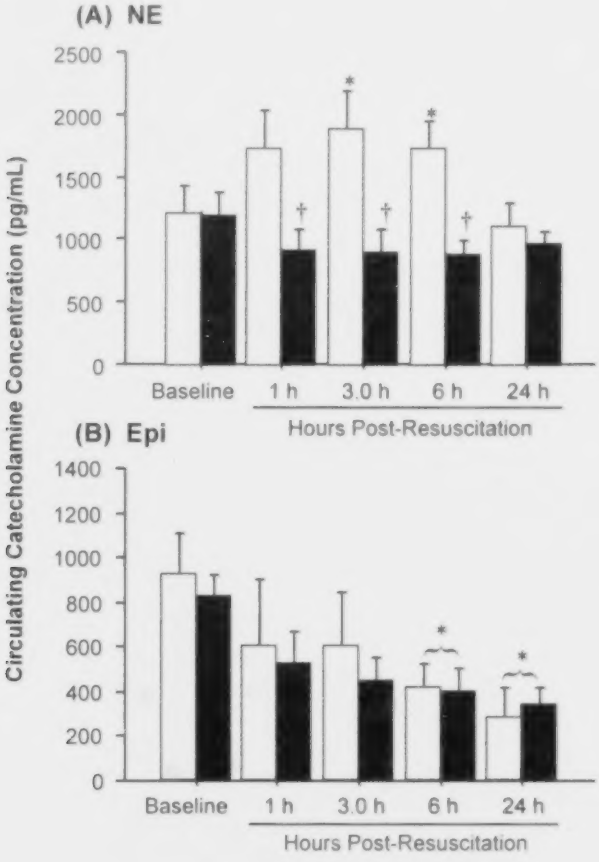

Figure 4: Changes in circulating catecholamine concentrations of resuscitated trauma patients. Normal saline (*NS*, □) and hypertonic saline-dextran (*HSD*, ■)-treated patients exhbit unique hormone secretion profiles. Data are expressed as the mean (± SE) circulating concentration of norepinephrine (*A*) and epinephrine (*B*). Significant differences: **P* < .05, post-resuscitation vs. baseline values within a treatment group; †*P* < 0.05, *HSD* vs. time-matched *NS* control values.

4.0 DISCUSSION

A growing awareness of the limitations of conventional isotonic crystalloid resuscitation fluids and the potential advantages of hypertonic/hyperoncotic solutions, has led to the reemergence of experimental and clinical interest in the role and mechanism of hypertonic saline resuscitation of hemorrhagic shock [9]. Increasingly, experimental evidence indicates that treatment with hypertonic saline/dextran (*HSD*) exerts profound immunoinflammatory activities, influencing post-injury cellular and molecular inflammatory reactions to shock/resuscitation leading to an improved host response [34,35]. The present clinical trial demonstrates, for the first time in resuscitated hemorrhagic shock patients, that compared with standard isotonic fluid therapy, supplementing initial resuscitation with a small-volume (250-mL) of *HSD* differentially modulates inflammatory cytokine and cellular adhesion molecule expression by blood monocytes of treated patients. Our results provide direct evidence that *HSD* infusion in the early stage following hemorrhagic shock helps reestablish the critical balance between pro and antiinflammatory mediators, which could lead to a reduction in post-traumatic complications and improved patient outcome as documented in animal models [5]. Additionally, this study suggests that *HSD*'s immunoregulatory activities are linked to altered sympathetic activation.

The pathophysiology of resuscitated hemorrhagic shock is characterized by an uncontrolled whole-body hyper-inflammatory reaction [8] that is triggered by a number of hostile stimuli, including, injured tissue, ischemia, hypoxia, and reperfusion damage [49]. Strong activation of the monocyte/macrophage system with excessive synthesis of the primary proinflammatory cytokines TNF-α and IL-1β, are thought to play a crucial role in the early pathogenesis of hemorrhagic shock [8]. These intercellular signalling molecules elicit their biological responses by activation of intracellular signal transduction pathways via binding cell-surface receptors, which are coupled to multiple downstream cytosolic intermediates and ultimately transmitted to nuclear regulatory factors (e.g., nuclear factor (NF)-κB), leading to the expression of target genes and regulation of target cell function [16]. Many normal physiologic processes, including, endocrine, immune and inflammatory responses are regulated by endogenous cytokine secretion. Therefore, homeostatic control of the balance between pro and antiinflammatory cytokines is critical for the maintenance of health [15]. Overwhelming insults such as severe hemorrhagic shock constitute a formidable challenge to the patient's ability to maintain this balance [13].

Blood monocytes are a heterogeneous population of mononuclear leukocytes that, like lymphocytes, comprise phenotypically and functionally distinct subsets [50]. They play a pivotal role in the innate immune response, serving as nonspecific effector cells, secreting cytokines and regulating tissue inflammation. At least two monocyte subpopulations are distinguishable on the basis of their expression of membrane antigens, migratory properties and cytokine production profiles [42,43]. In healthy individuals, the majority of circulating monocytes are strongly CD14-positive, but lack coexpression of CD16 ($CD14^{++}CD16^{-}$, classical monocytes). A minor subpopulation coexpress CD16 and are weakly CD14-positive ($CD14^{+}CD16^{+}$); these cells usually account for about 5–10% (~0.05 $x10^{9}$ cells/L) of all circulating monocytes under normal physiological conditions [43]. A significant finding of the present study was the marked differential redistribution of blood monocyte subpopulations observed in *HSD*-resuscitated trauma patients, as compared with patients who received just standard isotonic resuscitation.

$CD14^{+}CD16^{+}$ cells constitute a proinflammatory subtype that exhibit several features of inflammatory tissue macrophages, including, a distinct pattern of cytokine expression as compared with classical monocytes [43,50]. Specifically, the $CD14^{+}CD16^{+}$ subset have an enhanced capacity for TNF-α production [51], while they produce little or no IL-10 [44]. Circulating levels of $CD14^{+}CD16^{+}$ monocytes are known to increase dramatically (up to 50% of total monocytes) in patients with severe infectious diseases or other clinical

disorders with features of systemic inflammation [43,45]. Distinct alterations in the phenotype and function of blood monocytes have also been noted in patients undergoing major surgery [52]. Here, we demonstrate that both the mean proportion (~23%) and absolute counts (~0.12 x10^9 cells/L) of circulating $CD14^+CD16^+$ monocytes were already elevated in patients upon arrival at the trauma centre. Those patients resuscitated by standard methods experienced a continued expansion of the inflammatory subset (up to 35%) during the subsequent post-resuscitation sampling period. Remarkably, *HSD*-resuscitated patients displayed a significant reduction in $CD14^+CD16^+$ cells (<15%) over the same time-course. These results show clearly that *HSD* prevents post-traumatic expansion of the proinflammatory $CD14^+CD16^+$ monocyte phenotype. Considering that the frequency of $CD14^+CD16^+$ monocytes varies in association with several inflammatory states, and is directly related to the cytokine production potential of blood monocytes, our findings strongly suggest that *HSD* has the capacity to modulate monocytic inflammatory cytokine production.

Although the mechanism for this distinct pattern of monocyte subset redistribution is presently unknown, it can be speculated that enhanced post-traumatic sympathetic activation may be involved [19], since $CD14^+CD16^+$ monocytes are known to be selectively mobilized from the marginal pool in a rapid catecholamine-dependant fashion after extreme forms of physical stress [53]. The current study also demonstrates that *HSD* resuscitation elicits significant alterations in cell-surface expression of L-selectin (CD62L,) and β_2 integrins (CD11b) by circulating monocytes. Most notably, initial treatment with *HSD* was found to significantly blunt post-resuscitation expression of these adhesion molecules. Previous clinical studies, involving polytrauma patients, have shown that acute up-regulation of monocytic CD62L and CD11b occurs after injury and is related to the development of post-traumatic organ failure [54]. Furthermore, it has been shown that under inflammatory conditions, up to 70% of alveolar macrophages are may be derived from circulating $CD14^+CD16^+$ monocytes [55]. This finding raises the possibility that standard resuscitation fluids may promote the egress of inflammatory monocytes from the blood into the lungs through their enhancement of the $CD14^+CD16^+$ phenotype. Thus, our results suggest that *HSD* treatment may help avert widespread inflammatory monocyte activation upon injury via its capacity to modulate monocyte trafficking and by limiting leukocyte-endothelial interactions in the early post-traumatic period. Further studies are required to definitively elucidate the mechanism of differential shock/resuscitation-induced monocyte redistribution.

The major finding of the present investigation substantiates that supplementing initial resuscitation with a small quantity of *HSD* not only selectively influences the mobilization of functionally heterogeneous monocyte subsets, but also differentially modulates their intracellular expression of pro and antiinflammatory cytokines. With respect to TNF-α, we found that standard resuscitation induced a marked increase in the spontaneous and LPS-stimulated cytokine-producing activity of blood monocytes. Impressively, treatment with *HSD* effectively halted the upregulation of TNF-α expression at the single-cell level. Detailed monocyte subset analysis indicated that the expanded $CD14^+CD16^+$ subset was principally responsible for the enhanced TNF-α production. These findings indicate that specific blood monocytes are an important source of enhanced TNF-α production in resuscitated hemorrhagic shock patients, and that *HSD* resuscitation down-regulates this excessive inflammatory response. Our results are consistent with a recent study by Belge *et al.* [51], which provided evidence that the minor population of $CD14^+CD16^+$ inflammatory monocytes is a major source of intracellular TNF-α production in humans. The current findings are also in accordance with previous clinical reports showing significantly elevated monocytic gene expression and systemic concentrations of TNF-α immediately following major surgery [56], severe trauma or hemorrhagic shock [57].

To our knowledge, there are no other clinical reports evaluating the effects of hypertonic resuscitation on cytokine production in humans; however, available data from experimental studies support the current findings. Several reports indicate that hypertonic saline effectively inhibits spontaneous and LPS-stimulated cytokine production by a variety of tissues following hemorrhagic shock and ischemia-reperfusion [58].

Recent studies using a two-hit model of shock resuscitation found that hypertonic preconditioning inhibits LPS-induced TNF-α production by isolated alveolar and peritoneal macrophages [37,46,59]. Moreover, studies using cDNA microarray analysis to characterize inflammatory gene expression in major organs and human leukocytes after shock/resuscitation reveal that manipulation of fluid tonicity modulates cytokine gene transcription, including TNF-α expression [60]. A strength of the current investigation is the use of intracellular flow cytometric analysis, which has the advantage over conventional cytokine assays, in that it allows the immunophenotype, frequency and functional characteristics of individual cytokine-producing cells to be determined simultaneously within heterogeneous whole-blood cell populations, thus avoiding many problems inherent to soluble cytokine measurements [41].

An appropriate balance between pro and antiinflammatory cytokines is critical in the resolution of many pathological inflammatory conditions [16]. If unchecked, sustained dysfunctional inflammation contributes to sequential multiorgan failure and is a major cause of late trauma deaths [6,12]. Therefore, the immune system closely regulates proinflammatory cytokine production via upregulation of counter-inflammatory mediators. IL-10 is a prototypical endogoenous antiinflammatory and immunosuppressive cytokine [61], which inhibits monocyte/macrophage activation and down-regulates the biosynthesis of TNF-α and IL-1β, while preventing their biologic actions via up-regulation of IL-1ra [17]. TNF-α is the primary inducer of IL-10 synthesis, and IL-10 downregulates its own production via an autoregulatory feedback mechanism [62]. The antiinflammatory and immunosuppressive actions of IL-10 derive from its capacity to inhibit the translocation of NF-κB, leading to reduced transcription of a number of inducible genes involved in immune and inflammatory responses [63]. IL-10 also decreases leukocyte adhesion and recruitment to sites of inflammation [61]. Together these mediators serve to limit the potentially injurious effects of excessive inflammatory reactions [17].

Our results show that specific blood monocytes from patients resuscitated with *HSD* have an enhanced capacity for IL-10 and IL-1ra production, as compared with patients resuscitated by standard means alone. We determined that antiinflammatory cytokine expression exhibits a monocyte subset-specific production profile. That is, IL-1ra expression was detected in significant amounts in both $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes subsets, whereas, enhanced IL-10 expression was confined exclusively to $CD14^{++}CD16^{-}$ cells. This observation agrees with earlier findings demonstrating that $CD14^{+}CD16^{+}$ monocytes do not produce significant amounts of IL-10 [44], while IL-1ra is more ubiquitously synthesized [64]. These findings are also in agreement with recent animal studies showing that alveolar and peritoneal macrophages exposed to hypertonic conditions have significantly upregulated IL-10 synthesis [37,46]. Collectively, these observations strengthen the hypothesis that hypertonic resuscitation may exert its beneficial effects through early upregulation of counter-inflammatory mediators.

Nevertheless, previous experimental and clinical studies have produced conflicting results concerning the beneficial versus deleterious effects of endogenously elevated or systemically administered IL-10 [65]. In a number of studies, increased circulating concentrations of IL-10 have been associated with adverse clinical outcome in patients with sepsis syndromes. However, several recent investigations have shown that early therapeutic administration of IL-10 is effective in preventing the initial surge in TNF-α observed after traumatic hemorrhagic shock [66], and also in reducing the systemic inflammatory response and lethality in murine models of sepsis and reperfusion injury [67]. Indeed, Miller-Graziano *et al.* [68] suggested that trauma-mediated downregulation of IL-10 production contributes to the overproduction of TNF-α and increased occurrences of end organ failure in severely injured patients. Similarly, Donnelly and Colleagues observed that both IL-10 and IL-1ra were significantly lower in non-surviving ARDS patients [69]. These authors concluded that the failure of patients to mount an effective early antiinflammatory cytokine response contributed to the adverse outcome.

These results imply that when administered early in the post-trauma period, *HSD* has the capacity to rapidly up-regulate the counter-inflammatory cytokines, which are capable of attenuating hyper-inflammatory reactions, thereby preventing exacerbated pathology. It follows that initial resuscitation with *HSD* may also help avert a subsequent exaggerated immunosuppressive compensatory antiinflammatory response. This hypothesis is supported by laboratory evidence showing that *HSD* reduces the risk of delayed immunosuppression in mice after hemorrhagic shock by restoring suppressed lymphocyte function [70].

While the physiologic responses to *HSD* resuscitation are reasonably well defined [27], the mechanisms by which hypertonicity alters cellular and intracellular signaling pathways involved acute immunomodulation are not yet fully elucidated [11,35]. Potential targets include modification of various cell surface receptors, cytoplasmic second messengers and/or nuclear transcription factors [5,34]. Although the current study was not designed to directly evaluate the impact of hypertonicity on cytokine signaling, our finding that *HSD* greatly diminishes post-resuscitation concentrations of NE implies a sympathetic neuroendocrine mechanism for such alterations. The current findings also corroborate earlier studies showing that *HSD* resuscitation significantly attenuates the sympathoadrenal response to trauma and hemorrhage, with substantial reductions in circulating NE [71]. This is particularly relevant since catecholamines are recognized as key regulators of inflammatory cytokine production [18,20].

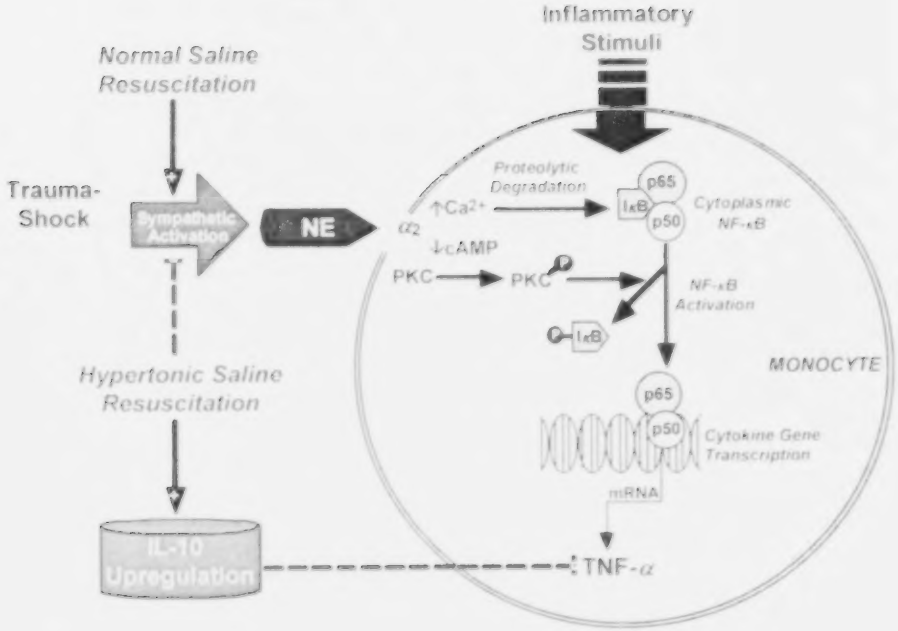

Figure 5: Potential sympathetic noradrenergic mechanisms of inflammatory cytokine modulation by hypertonic saline (*solid lines* indicate stimulation; *dashed lines* indicate inhibition; see text for details)

The adrenergic influence on cytokine production is highly complex, depending upon the setting, cell type and the receptor that is ligated [20]. In human monocyte macrophages, the vasoactive and immunological effects

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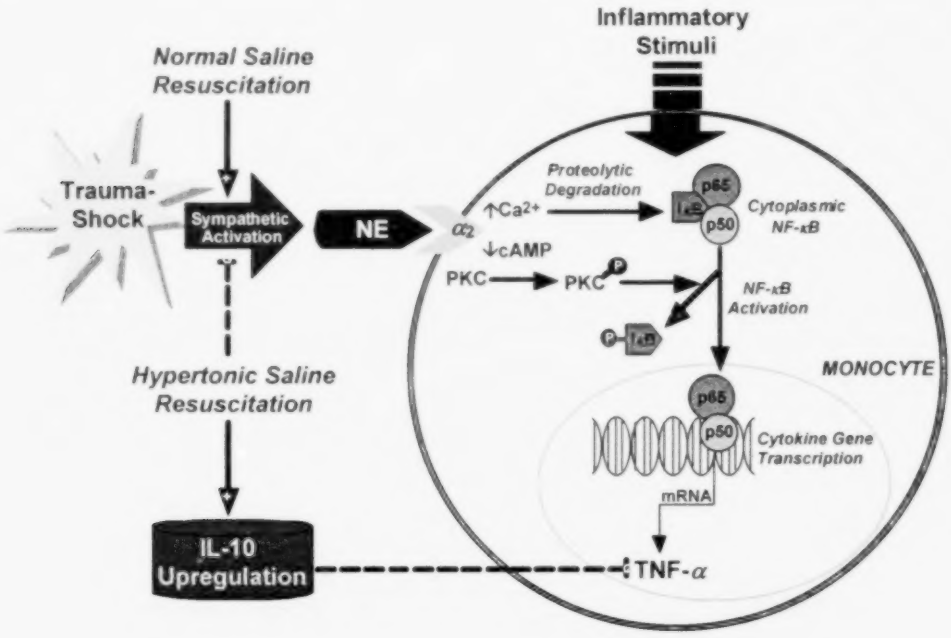

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of catecholamines are mediated via α- and β-adrenergic cell-surface receptors coupled to intracellular cAMP levels. NE-induced α-adrenergic stimulation has a cAMP-decreasing effect that enhances the formation of IL-10, but inhibits TNF-α [72] (see **Figure 5**). On the other hand, β-adrenergic stimulation by Epi mediates a cAMP-enhancing effect, which inhibits the production of TNF-α. Based on these observations and the present findings, it can be postulated that the upregulation of NE (mediating reduced cAMP) observed in patients resuscitated by standard means, contributes to excessive production of TNF-α and insufficient IL-10 induction in the early post-resuscitative period. Thus, it appears that through its specific capacity to inhibit NE secretion while preserving circulating Epi levels, *HSD* shifts the balance in favor of enhanced cAMP levels, thereby, mediating down-regulation of TNF-α and upregulation of IL-10.

The concept of *HSD*-induced differential sympathetic adrenergic cytokine regulation is supported by a number of experimental observations from previous studies. For example, in rats endogenous gut-derived NE has been shown to play a crucial role in the early systemic inflammatory response via enhanced hepatic α-adrenoreceptor stimulated TNF-α production [73]. Correspondingly, hemorrhage-induced pulmonary TNF-α expression is prevented by α-adrenergic blockade in mice [74]. Moreover, recent experimental findings demonstrate that exposure of human leukocytes to moderate hypertonicity triggers rapid accumulation of cAMP and suppression of their inflammatory activity [75]. Collectively, these findings suggest *HSD* resuscitation, through its capacity to stabilize shock/resuscitation induced NE concentrations, can dampen α-adrenergic-mediated TNF-α production, leading to reduced inflammation, ischemia and organ damage.

5.0 CONCLUSION

Hemorrhagic shock/resuscitation-induced immunoinflammatory alterations are clearly a complex phenomenon, likely due to the interaction of a variety of endogenous mediators. The present results demonstrate that, compared to standard treatment, supplementing initial resuscitation of hemorrhagic shock patients with 250-mL of *HSD* exerts a subset-specific immunomodulatory effect on peripheral blood monocytes, resulting in a shift in the dynamic balance between pro and antiinflammatory responses in the early post-resuscitation period. In particular, *HSD* seems to restore the balance between pro and antiinflammatory cytokines, by reducing initial TNF-α production by $CD14^{+}CD16^{+}$ monocytes, while simultaneously enhancing early antiinflammatory IL-10 and IL-1ra production by $CD14^{++}CD16^{-}$ monocytes. *HSD*'s beneficial therapeutic effects may, therefore, also derive from its ability to avoid extensive delayed counter-regulation by reducing the magnitude of the initial systemic inflammatory response. Although further research is required to identify specific intracellular mechanisms involved in *HSD*'s immunomodulatory actions, our results suggest that the differential cytokine expression in response to *HSD* may be mediated, at least partly, by preferential inhibition of post-resuscitation noradrenergic-induced TNF-α production and concomitant enhancement of IL-10 production via down-regulation of α-adrenergic-stimulated signaling pathways. These findings reinforce the notion that hypertonic/hyperoncotic solutions are not simply benign volume enhancers, but instead also act as potent pharmacological agents. As such, small-volume *HSD* resuscitation strategies should be designed to supplement, not supplant current fluid replacement modalities and prompt surgical intervention. Overall, *HSD* offers new promise as a simple, yet highly effective therapeutic approach for prevention of the excessive hyper-inflammatory state associated with resuscitated hemorrhagic shock and the risk of organ damage. These findings should influence the design of future clinical trials of hypertonic solutions in pre-hospital and hospital trauma care.

Acknowledgements

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